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The p53 tumor suppressor gene is mutated in about half of all human cancers and in roughly 30-40% of breast cancers. In order to better understand the role of p53 mutation and loss in breast cancer progression, we have developed a mouse model which is genetically programmed to develop mammary cancer in the presence and absence of p53. By comparison of the mammary tumorigenesis process between the p53 positive and p53 negative animals we hope to obtain further insights into the mechanisms by which loss of p53 accelerates tumor progression. In the four years of this grant we have shown that in the absence of p53 mammary tumors arise sooner and grow faster than mammary tumors with intact p53. We have also shown that tumors without p53 have higher levels of chromosomal instability and higher rates of cell proliferation than tumors with p53. Rates of apoptosis (programmed cell death) and angiogenesis (tumor vascularization) were not significantly different between p53 positive and negative tumors. We have examined the role of the p53-inducible cyclin-dependent kinase inhibitor p21 in mammary tumor progression and have shown that reduction of p21 accelerates tumor cell proliferation rates. Thus, the model is useful in elucidating the role of p53 loss in tumorigenesis and indicates that p53 has multiple roles in prevention of tumor formation and progression.

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Lawrence A. Donehower 10/1/98

PI - Signature Date

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Final Report: The p53-Deficient Mouse as a Breast Cancer Model

Principal Investigator: Lawrence A. Donehower, Ph.D.

Introduction:

The p53 tumor suppressor gene is mutated in roughly half of all human tumors examined to date (1). Approximately 30-40% of human breast cancers have p53 mutations, indicating loss of p53 function is a central event in breast cancer progression (1). Even in those breast cancers where p53 is wild type in structure, it may be abnormally stabilized or localized within the cell, suggesting possible disruption of p53-associated growth control pathways (2). In addition to its loss in spontaneously arising breast cancers, inherited mutations of the germ line p53 gene can also occur, giving rise to a familial cancer predisposition called Li-Fraumeni syndrome (3,4). The most frequently observed tumor in affected females of Li-Fraumeni families is breast cancer (5).

The role of p53 in the normal cell appears in large part to be that of a cell cycle checkpoint protein. In response to a variety of DNA damaging agents, p53 levels in the cell increase and mediate one of two cell fates: (i) arrest in G1 of the cell cycle, or (ii) apoptosis (6-8). The decision to undergo arrest versus apoptosis may rely on many factors, including cell type, growth factor levels, and abrogation of function in other growth-related genes, etc. Regardless of which decision is taken, the end result is to prevent the cell from DNA synthesis and division in the presence of damaged DNA templates (9). Thus, the cell is more likely to be spared oncogenic mutations which could lead to cancer. In the absence of p53 (which may occur in preneoplastic cells and does occur in p53-deficient mice), it is hypothesized that cells would be more likely to become tumorigenic through increased mutation rates and genomic instability (9).

The biochemical mechanisms through which p53 mediates its checkpoint functions are likely to be complicated, though clearly transcriptional regulation of key growth related genes is crucial for its G1 arrest function (8,10). Wild type p53 trans-activates the $p21^{WAF1/CIP1}$ gene, which encodes a potent inhibitor of G1 cyclin-dependent kinases (11,12). Transcriptional activation plays a role in p53-mediated apoptosis (13), though which p53 targets are critical for apoptosis are unclear. In other instances, it has been shown that p53 can mediate apoptosis in the absence of transcriptional activation, suggesting multiple mechanisms by which p53 affects cell death (14,15).

There is significant evidence that p53-mediated apoptosis may play an important role in suppression of tumors (16-18). Aberrant expression of certain oncogenes and tumor suppressor genes may induce high levels of p53 (19,20). When cells are induced to proliferate abnormally by these genes, p53 upregulation may induce apoptosis and thus protect the organism from early tumors. This role of p53 tumor suppression through apoptosis has been demonstrated in some mouse tumor models. In situations where Rb function is abrogated in a tissue, G1 arrest capability is

often lost and these abnormally proliferating cells are induced to undergo apoptosis. In the absence of p53, such Rb-deficient tissues may rapidly form aggressive tumors, arguing that attenuated apoptosis due to p53 loss is a rate limiting step in tumor formation (16-18).

Is apoptotic function the primary mechanism by which p53 regulates tumor progression or are there other mechanisms? A number of *in vitro* and *in vivo* studies suggest that loss or mutation of p53 may have additional important biological effects on tumor formation and progression (21). Some of these effects of p53 loss on tumor progression may be: (1) increased rates of cell proliferation independent of apoptotic effects; (2) increased levels of genomic instability in the tumor cells which may lead to further oncogenic lesions; (3) increased rates of angiogenesis, allowing more nutrients to nascent tumor cells; and (4) increased invasiveness and metastases. All of these biological effects are measurable, and an important goal of this proposal is to assess all of these potential tumor progression mechanisms in the context of the *Wnt-1*/p53 tumor model described below.

Initially, to study the role of p53 in tumorigenesis, we developed a p53-deficient mouse by gene targeting methods (22). These mice contained either one (p53+/-) or two (p53-/-) inactivated p53 germ line alleles. In comparison to their normal littermates, the p53-deficient mice showed accelerated tumorigenesis. Half of all p53+/- mice developed tumors by 18 months of age, while 100% of p53-/- mice succumbed to tumors by 10 months of age (23,24). The spectrum of tumors was quite variable, though lymphomas and sarcomas were most frequently observed (23,24).

While the analysis of tumorigenesis in the p53-deficient mice has yielded a number of interesting insights, the study of spontaneous tumor formation in these animals has certain limitations if one wants to examine mechanistic questions in an efficient, well controlled manner. For example, the p53-deficient mice develop a wide array of tumors sometimes with a relatively long latency (particularly the p53+/- mice). Moreover, since p53+/+ mice rarely develop tumors, control tumors which develop in a p53-independent manner are difficult to obtain. To circumvent these disadvantages, investigators have taken two general approaches, either treating the p53-deficient mice with a tissue-specific carcinogen or crossing the p53-deficient mice to a tumor-susceptible transgenic mouse genetically programmed to develop a single tumor type. The resulting models usually develop a single tumor type in a relatively short amount of time and control p53+/+ tumors are available to compare to the p53-deficient tumors.

The model we chose for examination of the role of p53 loss in tumor progression was the *Wnt-1* transgenic/p53-deficient mouse (25). This model was generated by crossing our p53-deficient mice to mammary tumor susceptible *Wnt-1* transgenic mice (26). *Wnt-1* transgenic females, which contain a mouse mammary tumor virus long terminal repeat promoter driving the *Wnt-1* oncogene, specifically develop early mammary gland hyperplasia followed by mammary adenocarcinomas before 12 months of age in a stochastic manner (26). Thus, our prediction was that virtually all of the *Wnt-1* transgenic females would develop mammary adeno-carcinomas either in the presence or absence of wild type p53. Such mice would be

ideal for exploring the biological and genetic effects of p53 presence and absence in mammary tumor formation and progression.

In the third and fourth years of our grant, we have supplemented our *Wnt-1* transgenic p53-deficient model with a second model, the *Wnt-1* transgenic p21-deficient mouse. We hypothesized that since an important effect of p53 in tumors was to reduce tumor cell proliferation, one possible molecular pathway that p53 inhibited proliferation was through induction of $p21^{WAF1/CIP1}$(11,12). We postulated that reduction or elimination of p21 in the *Wnt-1* transgenic mouse might have an effect on tumor progression comparable to that observed in p53-deficient *Wnt-1* transgenic mice.

p21 was first identified in 1993 as a cyclin dependent kinase inhibitor inducible by p53 (11,12,27-29). This protein was found to be associated with inactive cyclin E/CDK2 complexes that normally mediate G1 to S phase transition, indicating that the primary role of p21 is to effect G1 arrest (11,27,29). Overexpression of p21 in proliferating cells does indeed result in G1 arrest (30). Moreover, El Deiry et al. (12) demonstrated that p21 expression is transcriptionally activated by overexpression of p53, providing a potential mechanism through which induced p53 can mediate G1 arrest. Basal p21 expression is independent of p53, but following gamma irradiation increased expression of p21 is partially p53 dependent (31). p21 induction has been observed in cell lines undergoing induction of differentiation or senescence (31,32). p21 also binds PCNA and thus may inhibit DNA replication (33,34).

p21-deficient mice have been generated and p21 nullizygous mice have been shown to be developmentally normal (35,36). p21-deficient mice also do not appear to be tumor prone, indicating that p21, while a cell cycle inhibitor, is not a tumor suppressor in the biological sense. This is corroborated by a paucity of p21 mutations in human tumors (37,38). Interestingly, however, embryo fibroblasts from p21 null mice do appear to have enhanced division capabilities and show some defects in G1 arrest following treatment with DNA damaging agents (35,36).

Recent work by LaBaer et al. (39) have revealed that the assembly of G1 cdk/cyclin complexes is actually promoted through binding to p21. Moreover, there appears to be interesting stoichiometric effects based on the level of p21 which binds to the cdk/cyclin complexes. High levels of p21 inhibit the cdk/cyclin complex kinase activity, while absence of p21 results in low kinase activity (probably through failure of cdks and cyclins to form an active complex). However, low to intermediate levels of p21 appear to provide enough activity to promote formation of cdk/cyclin complexes, but not enough to actively inhibit the complex kinase activity. The end result of low to intermediate levels of p21 is enhanced cdk/cyclin activity and enhanced progression of the cell cycle through G1. This model was directly tested in our p21/*Wnt-1* crosses.

Body:

Experimental Methods

Mice

The *Wnt-1* mice used in the crosses described here were the offspring of two *Wnt-1* males from line 303 described previously (26). These mice were of mixed SJL X C57/BL/6 genetic background. The p53-deficient mice were from a pure 129/Sv line of mice containing one or two germ-line *p53* null alleles (40). The two *Wnt-1* males were crossed to heterozygous (*p53*+/-) 129/Sv females to derive F1 mice of four possible genotypes (*p53*+/+; *Wnt-1 p53*+/+; *p53*+/-; *Wnt-1 p53*+/-). F1 *p53*+/- females were crossed to F1 *Wnt-1 p53* +/- males to obtain F2 mice that carried any of the *Wnt-1 p53* genotypes found in the F1 population as well as *Wnt-1 p53*-/- or *p53*-/- . To obtain larger numbers of mice with *p53*-/- genotypes with or without the *Wnt-1* transgene, F2 *p53*-/- females were mated to *Wnt-1 p53* -/- males. All of the mice were monitored visually twice weekly for the appearance of tumors for up to one year. When a tumor of roughly 0.5 centimeters in diameter was detected, the age of the mouse was recorded and used to generate the Kaplan-Meier plots in Fig. 1. After appearance, the growth of the tumor was monitored by measurement in two dimensions of the diameter with a set of calipers. Once a tumor reached 2-2.5 cm in diameter, the tumor-bearing mouse was sacrificed, and tissue sections removed for histopathology. The rest of the tumor was frozen at -70°C for nucleic acid analyses.

The p21-deficient mice used in this study were obtained from Phil Leder and have been described in Deng et al. (35). Female p21-/- mice were crossed to *Wnt-1* transgenic males and F1 mice were intercrossed in a manner similar to that described for the p53-deficient *Wnt-1* transgenic mice described above. Ultimately, when sufficient numbers of p21+/+, p21+/-, and p21-/- *Wnt-1* transgenic females were obtained, these were monitored for mammary tumor formation.

Genotyping of Mice

We determined the *p53* and *Wnt-1* genotypes of the offspring from the crosses using previously described methods (25,26). Briefly, 1 cm of tail was clipped from weanling mice and incubated overnight at 55-60° C in 500 µl of tail lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 100 mM NaCl, 5 mM DTT, 200 µg/ml Proteinase K). Lysates were extracted with phenol chloroform and precipitated in two volumes ethanol. Pellets were resuspended in 100 µl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Five µl of each tail DNA was cleaved with Bam HI (2-16 hr at 37° C) and subjected to agarose gel electrophoresis on a 0.7% gel. The gel was then blotted to nylon membranes (Bio-Rad Zetaprobe membranes) and hybridized according to the Southern blot hybridization procedures of Reed and Mann (41). *Wnt-1* probes and p53 probes were utilized simultaneously in the hybridization and were labelled with ^{32}P with an oligo labelling kit provided by Boehringer-Mannheim. Filters were rinsed and subjected to autoradiography. The p53 wild type allele (5.0 kb), mutant p53 allele

(6.5 kb), *Wnt-1* transgene (3.0 kb), and endogenous *Wnt-1* gene (2.0 kb) could easily be differentiated by this procedure.

p21 genotypes of p21-deficient *Wnt-1* transgenic mice were determined as described in Deng et al. (35).

p53 Loss of Heterozygosity Assays

These assays were performed by Southern blot hybridization procedures identical to those described above for genotyping except that small pieces of mammary tumor tissue were used instead of tail. Roughly half of the tumors displayed loss of the remaining wild type p53 allele while half retained it by Southern assay (25).

Histological sample preparation

Tumors were surgically removed after four weeks of growth and cross sections were cut with a sterile razor blade. These samples were then fixed overnight in Methacarn, followed by four hours in acetone and then stored in 95% ethanol until embedding. Samples were embedded in paraffin using standard methods. Tumor sections were cut with a microtome to a width of 4 μ and fixed on microscope slides. Some slides were stained with hematoxylin and eosin according to standard methods and other slides were left unstained.

Tumor Transplantation Assays

Either 10^4, 10^5, or 10^6 tumor cells isolated from primary mammary tumors were transplanted into the inguinal mammary fat pads of female SCID hosts 8-16 weeks of age. These tumor cells developed into tumors whose diameter and growth rates were measured by calipers over a period of 6 weeks. The tumor cells were isolated from the tumors by mincing with a sterile razor blade followed by a 3 hour incubation at 37°C in DMEM:F12, collagenase and antibiotics as described by Kittrell et al. (42). The tumor cells were spun down and then rinsed three times with PBS containing 5% ABS to inactivate the Collagenase. The cells were then resuspended in DMEM:F12 and an aliquot was counted on a hemocytometer. Approximately 10 μl containing the appropriate number of cells were then injected into the inguinal fat pad of female SCID hosts.

Mitotic Index Counts

A determination of the number of mitotic figures in each tumor section was performed as follows: Mitotic figures (cells containing obvious condensed chromosomes in various stages of mitosis) were counted in 10 random high power fields at or near the edges of standard hematoxylin and eosin stained tumor cross sections.

BrdU Incorporation

BrdU incorporation was performed using the Cell Proliferation Kit from Amersham (RPN 20). Briefly, 1 ml of BrdU (from kit) per 50 grams body weight of the mouse was injected intraperitoneally. The labeling of cells was allowed to proceed for three hours at which time tumors were harvested and fixed in methacarn fixative solution overnight. Samples were then transferred to acetone for at least three hours and then stored in 95% ethanol until embedding in paraffin. Sections were stained for BrdU incorporation as follows: Samples were deparaffinized by washing twice in xylene, twice in 100% ethanol and once in 70% ethanol. Samples were then rehydrated in PBS for 20 minutes. Endogenous peroxidase activity was quenched by immersing samples in 3% hydrogen peroxide for five minutes followed by two 10 minute rinses in PBS. The samples were then incubated at 37° C for one hour with nuclease and Anti-BrdU antibody (Kit), followed by a 20 minute wash with PBST and 10 minutes with PBS. This was followed by incubation with the secondary antibody for 30 minutes at 37° C. Samples were then washed for 10 minutes with PBST and twice for 10 minutes with PBS. Substrate was provided in the form of DAB in 1x PBS for 10 minutes. Counterstaining is done with Methyl Green for 5 minutes followed by dipping in butanol, 100% ethanol, and xylene. Samples were then mounted with Permount.

Flow Cytometry

The DNA content of tumor samples was assessed in nuclei isolated from paraffin embedded samples based on a modification of Hedley's method (43). Nuclear suspensions were prepared from five 50 μm sections, a 4 μm section was cut before and after the 50 μm sections to confirm that histologically comparable sections were being used. The samples were deparaffinized using two 50 minute xylene washes at 25° C. The cells were then rehydrated using successive incubations with ethanol, two per dilution, 100%, 95%, 70%, 50% and distilled water. Between each concentration of ethanol there is a 50 minute incubation at 25° C to make a cell suspension. To make this a single cell suspension the cells were treated with a 0.5% pepsin solution at 37° C for 30 minutes. Filtration through a 74 μm mesh (Small Parts Inc., Miami, FL) resulted in a nuclear suspension which was stained using the Vindelov technique (44). For a procedural control fresh chicken erythrocyte nuclei (CEN) (Accurate Chemical & Scientific Co., Westbury, NY) were used. A five microliter solution of a 20,000,000 CEN/ml suspension was added to the sample prior to the DNA staining step.

The sample used for actual DNA flow analysis was acquired of a FACScan flow cytometer using the ModFitLT software with doublet discrimination from Becton Dickinson, San Jose, CA. Twenty thousand events were collected using the gating parameters of FL2-W versus FL2-A. Histograms were generated to determine the percentage of cells in each stage of the cell cycle. The Coefficient of Variation (CV) was determined for the diploid G0/G1 peak for all cases. Only those samples with CV's of less than 8% were used.

DNA Fragmentation Assay

Genomic DNA was isolated from frozen tumor tissue by methods similar to those described for the loss of heterozygosity procedure except that pelleting of the DNA utilized 30 minute centrifugations rather than five minute centrifugations. Twenty micrograms of total DNA from each tumor was then run out on a 1.5% agarose gel. The gel was blotted using standard Southern blot procedures (28) and probed using a total mouse genomic probe cleaved with Hae III and labelled with ^{32}P through the oligo labelling procedure with the High Prime labelling kit (Boehringer-Mannheim). Following autoradiography of the Southern blot, the radioactivity on the filters was imaged and quantitated using a Molecular Dynamics phosphorimager. The amount of hybridization in the low molecular weight ladder bands (apoptotic fragments) of each lane was divided by the total amount of hybridizing radioactivity (after subtracting background) in that lane to obtain an estimate of the percentage apoptotic DNA.

TUNEL Assay

The TUNEL assay was performed using the TACS™ 2 TdT *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Briefly, 4 µm sections on slides were deparaffinized by heating at 57° C for 20 minutes followed by placing in xylene for 10 minutes at room temperature. This was followed by another 5 minute xylene wash, a 5 minute 100% ethanol wash, a 5 minute 95% ethanol wash and a 70% ethanol wash for a further 5 minutes. The slides were then rinsed twice in distilled water for 2 minutes each followed by a rehydration in PBS for 10 min. The samples were then digested for 10 minutes at room temperature with Proteinase K (Kit). Endogenous peroxidases were then quenched using 3% hydrogen peroxide for 5 minutes. The fragmented DNA ends present in apoptotic cells were then extended using TdT, Mn^{++}, biotin dNTP's in labeling buffer (Kit). This reaction mix was added to the samples and allowed to incubate at 37° C for 1 hour. The reaction was stopped and rinsed for 2 minutes in PBS. Streptavidin-HRP conjugate was then allowed to bind to the incorporated biotin-dNTPs at room temperature for 15 minutes. Substrate was provided in the form of DAB and the color reaction was allowed to proceed for 5 minutes at room temperature. Counterstaining was performed for 5 minutes with 1.0% methyl green (Kit). Samples were dipped briefly in butanol, 100% ethanol and xylene. Finally, samples were mounted with Permount.

Immunoblot detection of antibodies in mammary tumors

Equivalent amounts of protein from mouse tumors were separated by SDS polyacrylamide gel electrophoresis using 15% resolving gels, transferred to Immobilon P membrane (Millipore), and immunoblotted with either anti-mouse IgG (H+L) antibodies (Pierce), mouse kappa or lambda chain-specific antibodies (Cappell), or tumor bearing serum as previously described (45,46).

Telomerase, telomerase RNA, and telomere assays

The procedures for these assays are described in Broccoli et al. (47).

Angiogenesis assays

Blank slides containing 5 micron sections of tumor tissue were deparafinized by 10 minute and 5 minute incubations in xylene, following by 3 minute sequential incubations in 100% ethanol, 95% ethanol, 70% ethanol, and three incubations in phosphate buffered saline (PBS), one 10 minute incubation in 3% hydrogen peroxide, and two 2 minute washes in PBS. Antigen retrieval was then performed by 15 minute incubation in Proteinase K solution (1 µl enzyme in 50 µl water from the Trevigen Apoptosis Kit), followed by three washes for 5 minutes in PBS. Blocking was performed by incubation with 10% goat serum in PBS for 30 minutes. Then, the primary Factor VIII antibody (A-0082 from DAKO; 1:200 dilution in 10% goat serum) was incubated on the slide overnight at 4° C in a humidified chamber. The slide was equilibrated to room temperature, and washed three times in PBS (the first wash contained 2% Tween). The secondary biotinylated antibody (DAKO E-0432) was then added (diluted 1:300 in blocking solution) for 45 minutes at room temperature in a humidified chamber. The slide was then washed three times by five minute incubations in PBS (the first wash contained 2% Tween). The slide was then incubated for 30 minutes at room temperature with the ABC reagents (streptavidin peroxidase from Vector Labs), followed by three washes in PBS and incubation in diaminobenzidine (250 µl in 50 ml PBS, 30% H_2O_2 from Trevigen) for 13 minutes. The slide was then washed in PBS for one minute followed by 2 minutes in 1% methyl green and three washes for 30 seconds in butanol, ethanol, and xylene. Finally, the slide was mounted with Permount and visuallized at 200X magnification.

Tumor angiogenesis was quantitated by counting the number of stained capillaries in 10 random high powered fields at 200X magnification along the tumor edge. The mean value of number of microvessels per field was then determined. Ten tumors per p53 genotype group were investigated by this method.

Differential display

Initially, to perform differential display, we isolated mRNAs from 4 *Wnt-1*/p53+/+ tumors, 4 *Wnt-1*/p53+/- LOH tumors, and 4 *Wnt-1*/p53-/- tumors using the Invitrogen mRNA purification kit according to the manufacturer's specifications. cDNAs were then synthesized from these 12 mRNAs using the Clontech Delta RNA Fingerprinting Kit according to the manufacturer's specifications. For each of the 12 cDNAs, one of ninety primer pair combinations provided by the Clontech kit was used to amplify the cDNA through 25 PCR cycles according the Clontech specifications. Each reaction contained alpha ^{33}P-dATP to label the amplified fragments. Each of the 12 amplification reactions were then loaded on a 5% denaturing polyacrylamide gel and electrophoresed. Autoradiography revealed a series of bands for each tumor mRNA which were almost always identical for all twelve tumors. Occasionally, differentially expressed bands were observed for a single tumor. These were usually ignored.

However, if differentially expressed bands were observed among three or four tumors of a given given genotype, these bands were isolated from the gel and reamplified with the same primers used initially.

To confirm that the differentially PCR fragments were indeed differentially expressed, candidate reamplified DNAs were labelled with ^{32}P by oligo labelling (Boehringer Mannheim High Prime kit) and hybridized to a Northern blot containing 5 µg of 15 tumor mRNAs, five *from Wnt-1*/p53+/+ tumors, five from *Wnt-1*/p53+/- LOH tumors, and five from *Wnt-1*/p53-/- tumors. Following autoradiography, the comparative expression patterns were observed. In at least one case (out of four candidate fragments examined so far), the initial differential expression pattern was confirmed in the Northern blot analysis.

In Vitro Invasion Assay

Matrigel chambers from Becton-Dickinson were rehydrated with DMEM:F12 medium for two hours. Cell suspensions of 4.4 X 10^5 mammary tumor cells in DMEM:F12 with insulin were prepared. These cell suspensions were placed in the matrigel chamber insert. The insert was then placed ina six well companion plate containing DMEM:F12 with insulin, 2% serum, and EGF. Cell culture inserts were removed after a 24 hour incubation. Cells and matrigel were wiped from the top of the insert using a cotton swab. The bottom of the insert was then stained using a Diff-Quick staining kit from Dade Diagnostics. The insert membraned was removed with a razor blade and placed on a microscope and slide with immersion oil. Cells passing through the membrane were then counted at 200X magnification.

Immunoprecipitation-Western blot analyses

One and a half milligrams of tumor lysate was precleared with 15 µl each of protein A and G agarose (Boehringer-Mannheim) for 8 hours at 4°C. Samples were spun and removed from the protein A and G agarose. 5 µg of anti-cyclin D1 antibody (C-20, Santa Cruz) were added and allowed to bind 6 hours followed by addition of 50 ml of BioMag protein G (PerSeptive Biosystems). Samples were incubated at 4°C for a 8 hours. The complex was collected using magnetic stands (Promega) and washed two times with D1 lysis buffer and 50 mM HEPES pH 7.5. Sample buffer was added and samples were boiled for five minutes. Samples were run on a 15% SDS-polyacrylamide gel. p21 protein was detected with a mouse IgG monoclonal antibody to p21 (AB-4, Oncogene Research) for 12 hours. Blots were washed with Tris-buffered saline Tween followed by a four hour incubation with goat anti-mouse peroxidase (Boehringer-Mannheim). Protein was detected using the Supersignal ECL System (Pierce). Quantitation of band intensities was performed by densitometry with a Molecular Dynamics Personal Densitometer SI.

Rb kinase assay

The cyclin D1-associated complexes were isolated as described above except that 700 µg of tumor lysate was used for immunoprecipitation. After the overnight collection with magnetic beads samples were washed four times with kinase buffer as previously described (28). The kinase reaction was set up with 48 µl kinase buffer, 1 µl Rb substrate (Santa Cruz Biotechnology) and 1 µl gamma ^{32}P-ATP. Samples were incubated for 30 minutes at 30°C. Incubation sample buffer was added and samples were boiled for 5 minutes before running on an 8% polyacrylamide gel. Gels were dried and exposed to film. Kinase levels were quantitated using a Storm 860 Phosphorimager and ImageQuant data analysis software (Molecular Dynamics).

Results

(1) Results in first year of grant

These studies were initiated following a collaborative agreement with Dr. Harold Varmus to cross our p53-deficient mice with his *Wnt-1* transgenic mice that were susceptible to mammary tumors. I felt that mechanistic studies of the role of p53 loss in tumor initiation and progression would be greatly facilitated by a bitransgenic model in which (1) only one type of tumor is observed, (2) the tumors arise within a shorter span of time, (3) p53+/+ control tumors are readily available, and (4) tumors could be detected early and tumor growth rates could easily measured because of their subcutaneous location. The *Wnt-1* transgenic p53-deficient model fulfills these criteria quite well as only mammary tumors (regardless of p53 genotype) are observed in a reasonable span of time (100% mammary tumor development in females by 9 months of age). These mammary tumors can be observed when less than a half centimeter in diameter and tumor growth rates can be monitored by measuring by calipers the diameter of the tumor over a period of several weeks.

First, After crossing the p53-deficient mice to the *Wnt-1* transgenic mice, we monitored tumor formation in the various categories of offspring. We will focus on the *Wnt-1* transgenic females in the remainder of this report. Appearance of mammary tumors in the *Wnt-1* transgenic females was greatly accelerated in the absence of p53 (p53-/-) (Figure 1). All of the *Wnt-1* p53-/- females developed mammary adenocarcinomas by 15 weeks of age and very few developed lymphomas or other spontaneous tumors characteristic of the p53-/- mice. *Wnt-1* p53+/+ females showed a delayed mammary tumor incidence with 100% developing adenocarcinomas by 41 weeks of age. Interestingly, *Wnt-1* p53+/- females exhibited the same mammary tumor incidence as *Wnt-1* p53+/+ females, even in those cases where the p53+/- tumor showed loss of the remaining wild type allele.

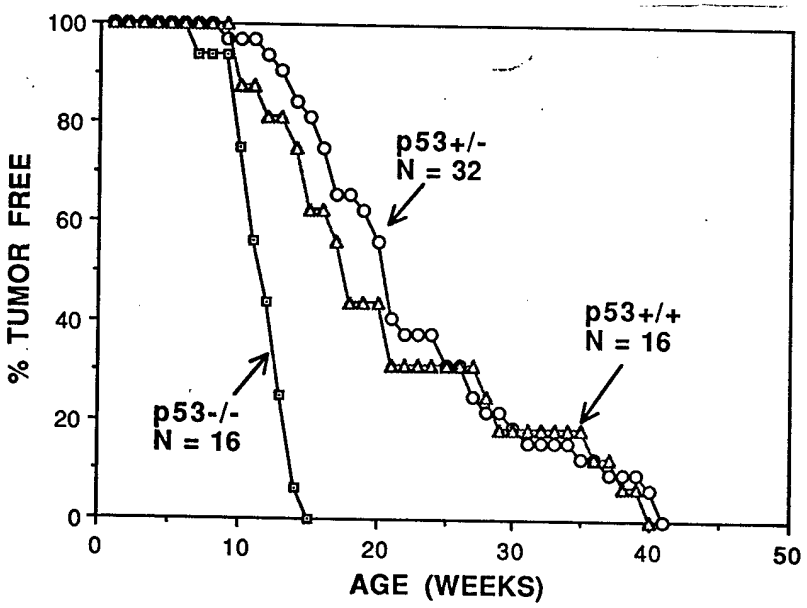


Figure 1. Mammary tumor incidence in *Wnt-1* transgenic 53-deficient female mice.

In order to obtain insights into the mechanisms by which loss of p53 accelerated tumorigenesis, we analyzed the chromosomal complement of the *Wnt-1*-initiated mammary tumors in the presence and absence of p53. Previous *in vitro* studies had shown that the loss of p53 correlated with genomic instability. Using two different methods, classical cytogenetics and comparative genomic hybridization, we showed that loss or absence of p53 correlated quite well with increased genomic instability. A summary of the comparative genomic hybridization (CGH) results is shown on the next page (Table 1). It should be noted here that roughly half of the p53+/- tumors lose their remaining wild type allele (referred to as p53+/- LOH) and roughly half of the tumors appear to retain their wild type allele (referred to as p53+/- no LOH) as assayed by Southern blot hybridizaton. Sequencing of the p53 cDNA in four p53+/- tumors which retained the wild type allele showed no point mutations in the remaining p53 gene and no downregulation of RNA expression, suggesting that wild type p53 function is retained in this category of p53+/- tumors.

Table 1. Summary of CGH Abnormalities in *Wnt-1* Mammary Adenocarcinomas

p53 Genotype	Tumors with Chromosome Abnormalities	Average Number of Chromosome Abnormalities per Tumor
p53+/+	2/6	0.3
p53+/- (no LOH)	2/4	1.0
p53+/- (LOH)	8/8	4.2
p53-/-	7/7	1.7

Tumors missing p53 (p53+/- LOH and p53-/-) showed significantly higher rates of chromosomal abnormalities, supporting the argument that loss of p53 promotes genomic instability. Surprisingly, Table 1 also shows that p53+/- tumors which lose their remaining wild type p53 allele have even more chromosomal abnormalities than p53-/- tumors. We are unsure why this would be so, but have speculated that p53+/- tumors have to undergo more mutations than p53-/- tumors because of environmental effects (i.e. organisms with no p53 may not be able to express certain extracellular inhibitors of tumor growth). Interestingly, the chromosomal abnormalities observed in the p53-deficient tumors were non-random in nature, suggesting a selection for certain chromosomal lesions which might provide a growth advantage. These results indicate that genomic instability does play a role in the progression of these tumors and that loss or absence of p53 may contribute to tumor progression in part through this mechanism.

(2) Results in second year of grant

In the second year of the grant our primary focus was to determine some of the biological mechanisms by which loss or absence of p53 accelerates mammary tumorigenesis in the *Wnt-1* transgenic p53-deficient model. In the first year we concentrated on the involvement of genomic instability in tumor progression. In the second year we looked at three potential additional mechanisms: (i) cell cycle progression and proliferation; (ii) apoptosis (programmed cell death); and (iii) telomere and telomerase effects. The first two parameters are obviously of critical importance in the growth of the tumor as the tumor growth is a direct function of the rate of cell division minus the rate of cell death. There has been significant evidence from other tumor models that loss of p53 is accompanied by attenuated apoptosis and that this event might be a rate limiting step in tumor formation (16-18).

(a) mammary tumor growth in the presence and absence of p53

Our first set of studies entailed some followup experiments on the tumor incidence data shown in Figure 1. The data in Figure 1 measures the time to first appearance of a visible tumor. Such curves may reflect early events in tumor formation. However, we wanted to more effectively measure the rates of tumor growth once the tumor is first observed. To do this, we measured the rate of tumor volume growth over time after initial observation. Tumor diameters in two dimensions were measured with calipers at weekly intervals and tumor volumes could then be estimated from these measurements. We found that while the rate of tumor volume growth for each p53 genotype was quite variable, the average growth of tumors missing p53 was faster than those containing p53 (Figure 2).

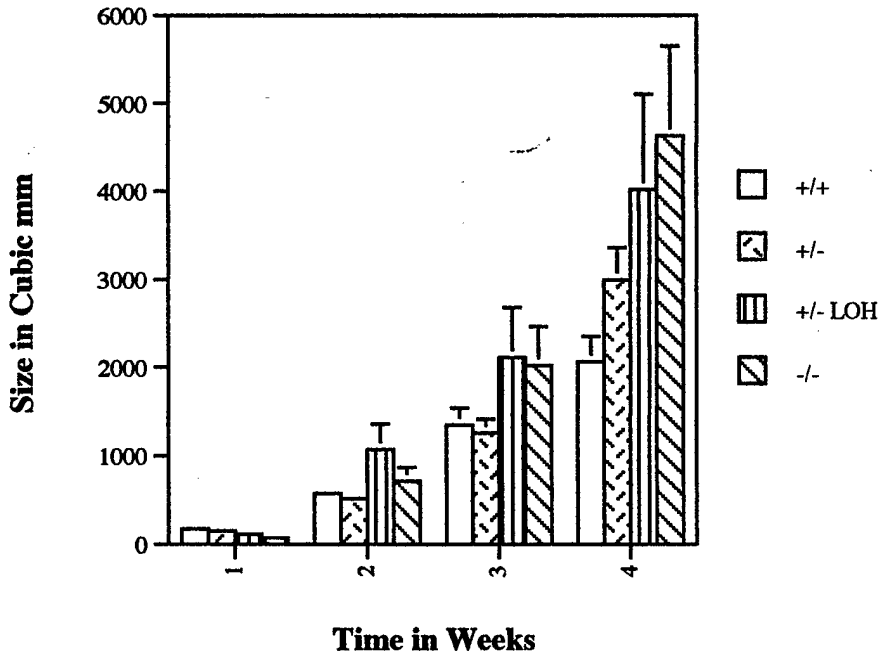


Figure 2. *Wnt-1* transgenic mammary tumor growth rates in the presence and absence of p53.

We also performed tumor transplantation experiments in which we transplanted 10^5 tumor cells from each tumor and injected them into the same inguinal mammary fat pad of genetically indentical recipient SCID mice. Virtually all of the transplantations with 10^5 tumor cells formed tumors which grew gradually enough to easily differentiate between fast growing and slow growing tumors. We started with the same number of tumor cells (10^5), injected into the same inguinal mammary fat pad, in genetically identical p53+/+ female SCID hosts between 8 and 16 weeks of age. The results did not show the expected straightforward correlation between growth rate and p53 status. However, the p53+/+ tumors did show the slowest growth rates, and the p53+/- LOH tumors, after a slow start, had the fastest growth rate. Interestingly, the p53-/- tumor cells, which showed the fastest growth rate as primary tumors, showed slower growth rates than even the p53+/- no LOH tumors. We speculated that such slow rates were attributable to an environmental effect. That is, organisms with intact p53 (such as the p53+/+ SCID hosts in these experiments) may release an inhibitor of tumor growth which p53-/- tumor cells have never been exposed to in the primary tumor. However, these experiments partially corroborated the primary tumor data in that the absence or loss of p53 tended to result in an increase in mammary tumor cell growth rates.

(b) tumor cell proliferation and cell cycle progression

Probably the most obvious mechanism by which p53 loss could result in elevated tumor cell growth is through increased rates of cell division. We had shown previously that early passage p53-/- embryo fibroblasts divide more rapidly than their p53+/+ counterparts and have higher percentages of cells in S phase (48). To assess rates of cell division in the mammary tumors in the presence and absence of p53, we employed three different techniques: (i) mitotic index determinations; (ii) BrdU incorporation assays; and (iii) flow cytometry on fixed tumor cells. The mitotic index for each tumor was easily determined by counting the percentage of cells in a tumor in multiple microscopic fields which are clearly in mitosis through the identification of condensed chromatids in the fixed H & E sections. Two individuals performed these counts in a blinded fashion and obtained similar results. These are shown in Table 2.

Table 2. Mitotic Index of *Wnt-1* Mammary Adenocarcinomas

p53 Genotype	Mean Mitoses[a]	# Tumors Examined
p53+/+	16.7	9
p53+/- (No LOH)	23.8	6
p53+/- (LOH)	40.0	7
p53-/-	37.0	10

[a]Mean number of mitoses per ten high power fields

The results above clearly indicate that cells which either lack or lose p53 show higher frequencies of mitosis as measured by this assay. To confirm that tumors without p53 also have higher percentages of cells in S phase, we employed a BrdU incorporation assay. BrdU was injected into the tumor bearing animals three hours prior to sacrifice. Tumor cells in S phase during this three hour window were detected by incubation of the tumor sections with an anti-BrdU antibody. When the results were carefully quantitated, we again found that the p53+/- LOH and p53-/- tumors had higher percentages of cells in S phase than p53+/+ and p53+/- no LOH tumors.

Our final assay for cell proliferation involved flow cytometry on tumor cells from fixed sections of tumors to determine the DNA index and S-phase fraction. 23 *Wnt-1*/p53 tumors were examined by flow cytometry (49). As expected, tumors losing or missing p53 had higher fractions of cells in S phase. p53+/- LOH showed significantly higher S phase fractions than all other p53 genotypes. This result is consistent with the earlier mitotic index data. Interestingly, flow cytometry indicated that all five p53+/- LOH tumors were aneuploid while none of the other twelve tumors (including p53-/- tumors) appeared to have significant aneuploid fractions. This result was also consistent with comparative genomic hybridization data.

(c) apoptosis

The growth rate of a tumor is likely to be influenced by at least two processes, the rate of cell division and the rate of cell death. We have shown strong evidence above that in the *Wnt-1* mammary tumor model the absence of p53 increases cell divison rates over those seen in tumors with intact p53. However, studies on other mouse tumor models have indicated that loss of p53 in a tumor cell may be accompanied by attenuated apoptosis and increased survival (16-18). To determine whether p53 loss was accompanied by decreased apoptosis in our *Wnt-1*/p53 model we analyzed apoptosis by two methods, the TUNEL assay and DNA fragmentation assay. The TUNEL assay specifically labels nuclei with free DNA ends. Such free ends are signature events in apoptotic cells. We employed this assay on both normal and hyperplastic mammary glands and mammary tumors from the *Wnt-1*/p53 mice. Apoptosis levels were very low in both normal and hyperplastic mammary glands in the presence and absence of p53 (49) When mammary tumors were examined by TUNEL assay, *Wnt-1* tumors had low levels of apoptosis in both p53 positive and negative tumors and there was not a significant difference among the tumors (49).

p53 status plays little or no role in levels of apoptosis in the tumors in this model and this was further confirmed by a DNA fragmentation assay on the tumors. With this assay, there was not a great difference in the intensity of apoptotic DNA (represented by the discrete bands in the low molecular weight region of the gel following quantitation with the Molecular Dynamics Phosphorimager). If anything, we noted that p53-/- tumors had marginally higher percentages of apoptotic DNA compared to p53+/+ tumors (49). Thus, our data supports the hypothesis that the increased tumor growth rate observed in the tumors lacking p53 is likely to be a result of increased proliferation rate rather than decreased apoptotic function.

(d) antibody deposition in *Wnt-1* mammary adenocarcinomas

In a collaborative effort with Wafik El-Deiry at the University of Pennsylvania School of Medicine, we investigated $p21^{WAF1/CIP1}$ protein expression in p53-deficient tumors and *Wnt-1* mammary tumors (46). Using primarily immunoblot assays, assessment of p21 in the tumors tended to be variable and inconclusive. However, it was quickly noted that tumors from mice which had intact p53 (e.g. *Wnt-1* p53+/+ mice) had high levels of mouse light and heavy chain antibody deposition within the tumor tissue. In contrast, mice without p53 (e.g. *Wnt-1* p53-/- mice) had very low levels of antibody deposition within the tumor. Six of seven *Wnt-1* p53+/+ tumors had high levels of antibodies, while six of seven *Wnt-1* p53-/- tumors had very low levels of antibodies (46). Moreover, sera from tumor-bearing *Wnt-1* p53+/+ mice recognized multiple antigens in extracts from their own tumors. Sera from tumor-bearing *Wnt-1* p53-/- mice had substantially reduced reactivity with tumor antigens in their own tumors.

(e) telomere length, telomerase, and telomerase RNA

In collaboration with Titia de Lange at the Rockefeller, we were fortunate in being able to examine the contribution of telomerase and telomere degradation to tumor initiation and progression in the *Wnt-1* transgenic p53-deficient model (47). Some models of cancer formation implicate activation of telomerase activity as a critical event in tumor formation, since normal human cells in culture show both low telomerase activity and decreasing telomere size with passaging (50). Immortalized human cells and tumor cells often show increased telomere size and high levels of telomerase. It is postulated that early stage tumors may lose telomere function due to progressive shortening (51). This may lead to activation of DNA damage checkpoints, followed by cell cycle arrest and apoptosis. In tumors that have lost the ability to detect uncapped chromosome ends (e.g. p53-deficient cells), telomere malfunction may lead to genomic instability and accelerated tumor progression (51).

To test these possibilities, we compared telomere length, telomerase RNA, and telomerase activities in the normal mammary glands and mammary adenocarcinomas of the *Wnt-1* transgenic p53-deficient mice (47). Interestingly, mouse telomere length did not greatly vary in length between normal and tumor tissue, irrespective of p53 status, suggesting that decreasing telomere length is not an issue in mouse mammary tumorigenesis. Telomerase RNA was increased in amount in the mammary tumors compared to normal mammary glands, but p53 status had no apparent effect on the telomerase RNA levels. The most dramatic result was the high levels of telomerase activity observed in mammary tumors but not in the normal mammary glands or hyperplastic mammary glands (Table 3). There was a slight effect of p53 status on telomerase activity in the mammary tumors. p53-/- mammary tumors had roughly two fold more telomerase activity than p53+/+ tumors. However, it is unclear whether this slight increase in telomerase activity may have a significant effect on tumor initiation and progression in the p53-/- mice. The most likely conclusion is that loss or absence of p53 has little or no effect on telomere status in mouse mammary tumors.

Table 3. Activation of telomerase in *Wnt-1* mammary tumors in mice with different p53 genotypes

Type of sample	Telomerase activity[a]
p53-/- normal mammary gland	
MG 1-/-	9.3
MG 2-/-	4.5
MG 3-/-	18.3
Median	9.3
p53+/+ mammary tumor	
W2	17
W10	11
W30	36
W134	54
W151	24
Median	24
p53-/- mammary tumor	
W98	100
W121	94
W154	56
W177	14
W184	55
Median	54

[a] Telomerase activity is expressed as relative specific activity normalized to a mouse J558 standard. Average percent activity was determined from two to six assays.

(3) Results in third year of grant

(a) angiogenesis assays in *Wnt-1* transgenic, p53-deficient mice

Recent studies have implicated p53 as a potential regulator of angiogenesis. Thrombospondin-1, an inhibitor of angiogenesis, has been shown to be activated by wild type p53 (52). Thus, loss of p53 may positively affect the ability of tumors to undergo neovascularization. To test this possibility, we performed immunostaining on sections taken from the *Wnt-1*/p53 tumors using an anti Factor VIII antibody. Factor VIII staining is frequently used as a marker for capillaries and vasculature. Thus, by examining the degree of Factor VIII staining in a tumor section, one can get an idea of the degree of vascularization of that tumor. We were successful in obtaining Factor VIII staining in our tumor samples, but when we attempted to quantitate the degree of staining, we were unable to detect significant differences in tumor capillary density by several methods of quantitation (Fig. 3). Thus, in this particular in vivo context, late stage tumors do not show evidence of p53-dependent differences in angiogenesis. However, this does not rule out the possibility that p53 may have an effect during early stages of tumorigenesis.

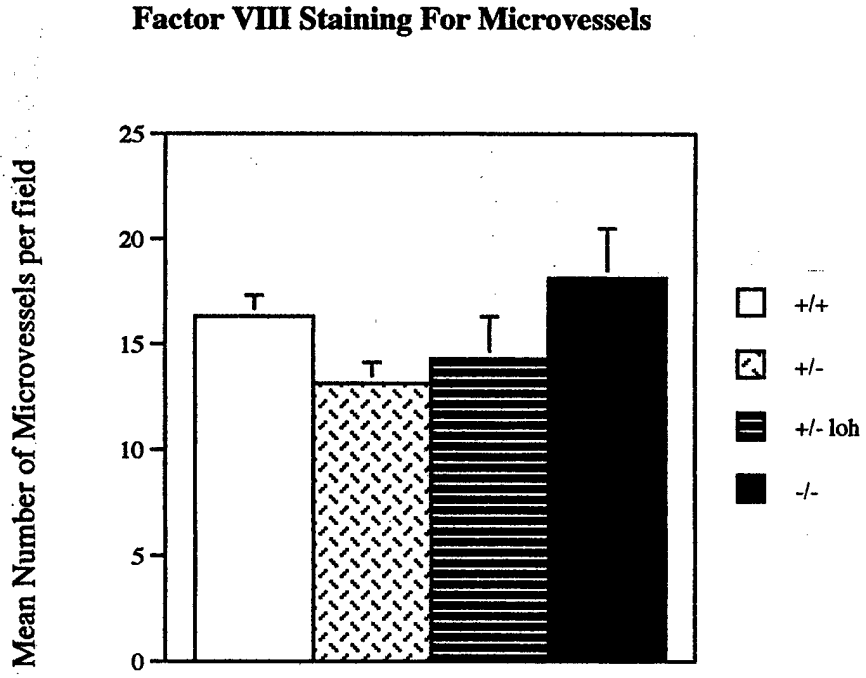


Figure 3. Mean microvessel density in *Wnt-1* transgenic tumors in the presence and absence of p53.

(b) invasiveness and metastasis assays

Evidence from other models suggested that p53 loss could potentially accelerate invasiveness and metastasis. To test this possibility in our Wnt-1/p53 model, we initially tested *Wnt-1*/p53+/+ and *Wnt-1*/p53-/- mammary tumor cells in the Marigel chamber in vitro invasion assay (Becton-Dickinson). Briefly, this assay

consists of placing cells on an artificial extracellular matrix overlaying a 8 micron porous membrane. The matrigel matrix serves as a reconstituted basement membrane in vitro. Those cells able to penetrate the matrigel and the porous membrane are considered invasive. Despite many efforts and changing a number of variables with our mammary tumor lines, we were unable to generate any significant numbers of invasive cells regardless of p53 genotype.

To assess metastasis in the *Wnt-1* transgenic model, we have examined the lungs of the tumor bearing mice for metastatic nodules. While metastases are relatively rare in the *Wnt-1* transgenic mouse, when they occur they usually arise in the lungs (H. Varmus, personal communication). So far, in comparing the p53+ and p53- tumors, we have failed to detect any differences in the rate at which the tumor bearing mice display any lung metastases in the presence and absence of p53. Thus, we have concluded that this model is not a particularly good one for studying the mechanisms of p53 in affecting metastasis.

(c) differential display to identify p53-regulated genes in tumorigenesis

Given the differences in tumor appearance and tumor progression in the p53+/+ and p53-/- *Wnt-1* transgenic mice, we thought it might be possible to detect p53-dependent differences in gene expression patterns through a differential display approach. Because p53 is a transcriptional regulatory protein which both activates and represses cellular genes, it is possible that at least some genes should show differential expression in the presence and absence of p53. We used the differential display approaches originally developed by Liang and Pardee (53,54). Identification of mRNAs expressed only in p53+ tumors or only in p53- tumors could provide insights into the biochemical mechanisms by which p53 loss accelerates tumor growth and progression. In addition, this approach may identify novel p53 target genes. This technique has been well publicized (53), so details of the methodology will not be reiterated here.

We have used several variations on this approach in our preliminary experiments, but have recently relied on the Clontech kit (Delta RNA Fingerprinting Kit) which appears to work very well. We have purified mRNA from p53+/+, p53+/- LOH and p53-/- *Wnt-1* mammary tumors (four separate tumors of each genotype) and subjected them to differential display analysis using the 90 different upstream and downstream primer combinations supplied in the kit. We used multiple tumors of each genotype because there is likely to be intertumor variation which is not dependent on p53. Only when we observed consistent differences between p53+ and p53- tumors did we proceed further and isolate the differentially expressed bands. Fragments which showed such p53 specific patterns were isolated from the gel and subjected to a further round of PCR prior to cloning in a vector designed for PCR fragments. We have completed the initial differential display analysis and have isolated 20 fragments which show evidence of consistent differences in the presence and absence of p53. Corroboration of the differential expression patterns has been initiated by Northern blot analysis of the mRNAs from the p53+ and p53- tumors. An example of one probe which has displayed p53-specific genotype differences is shown in Fig. 4.

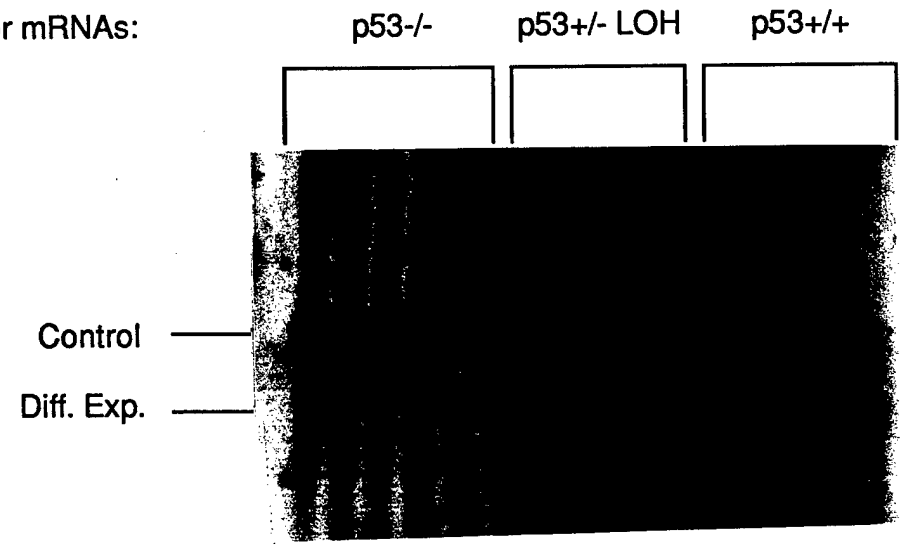


Figure 4.

Note that while all of the p53+/+ tumors demonstrate high levels of expression of this message, only 2 of 5 p53-/- tumors express this message, and at considerably lower levels than p53+/+ tumors. Moreover, the p53+/- LOH tumors also display decreased levels of this message, suggesting that this message is expressed in a partially p53-dependent manner. The upper band represents a control probe for loading which is expressed equally in all genotypes. Once we have identified all of the differentially expressed fragments by Northern blot analysis, the inserts will then be sequenced at the local sequencing core to determine their identity.

(d) tumor incidence and growth in *Wnt-1* transgenic p21-deficient mice

Recently, we have crossed the *Wnt-1* mice to p21 knockout mice obtained from Phil Leder. The *Wnt-1* transgenic female offspring of all three p21 genotypes have been examined for mammary tumor incidence and tumor growth rates. We found that while p21 status (p21+/+, p21+/-, or p21-/-) had little discernable effect on when the *Wnt-1* initiated mammary tumors arose, the growth rates of the tumors did show p21-dependent differences. p21+/+ tumors showed the similar slow growth observed in p53+/+ *Wnt-1* transgenic mice. However, the *Wnt-1*/p21+/- tumors showed dramatically higher growth rates than p21+/+ tumors, similar to the growth rates previously observed for *Wnt-1*/p53-/- tumors (Fig. 5).

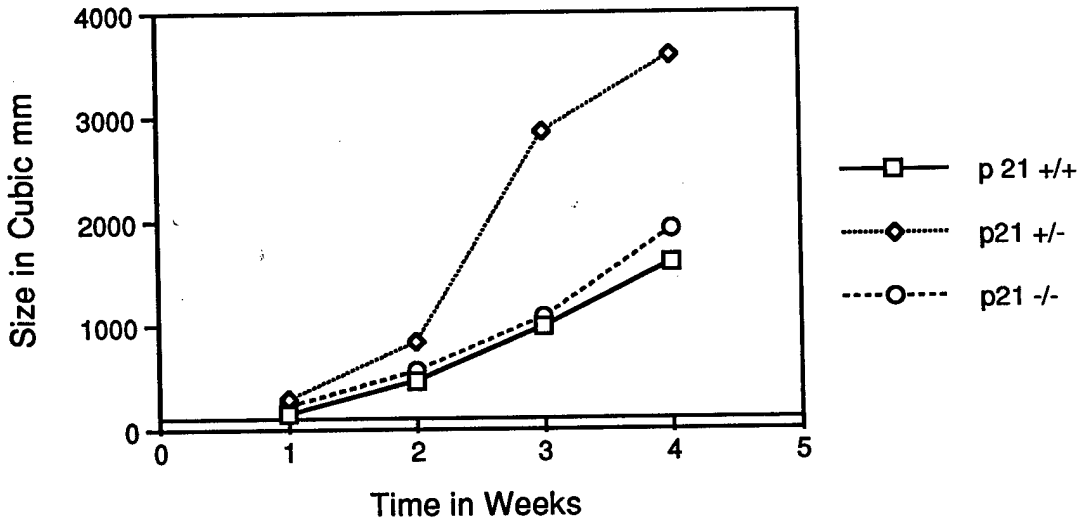


Figure 5.

Interestingly, the *Wnt-1*/p21-/- tumors showed growth rates equivalent to the *Wnt-1*/p21+/+ tumors. These growth rate differences were confirmed by mitotic index assays which demonstrated significantly higher fractions of p21+/- tumor cells in mitosis and S phase compared to p21+/+ and p21-/- tumor cells. Figure 6 shows the relative mitotic index for the *Wnt-1* transgenic tumors of all three p21 genotypes. While the differences in mitotic index are not as dramatic as in the tumor growth curves, they are significant by t-test.

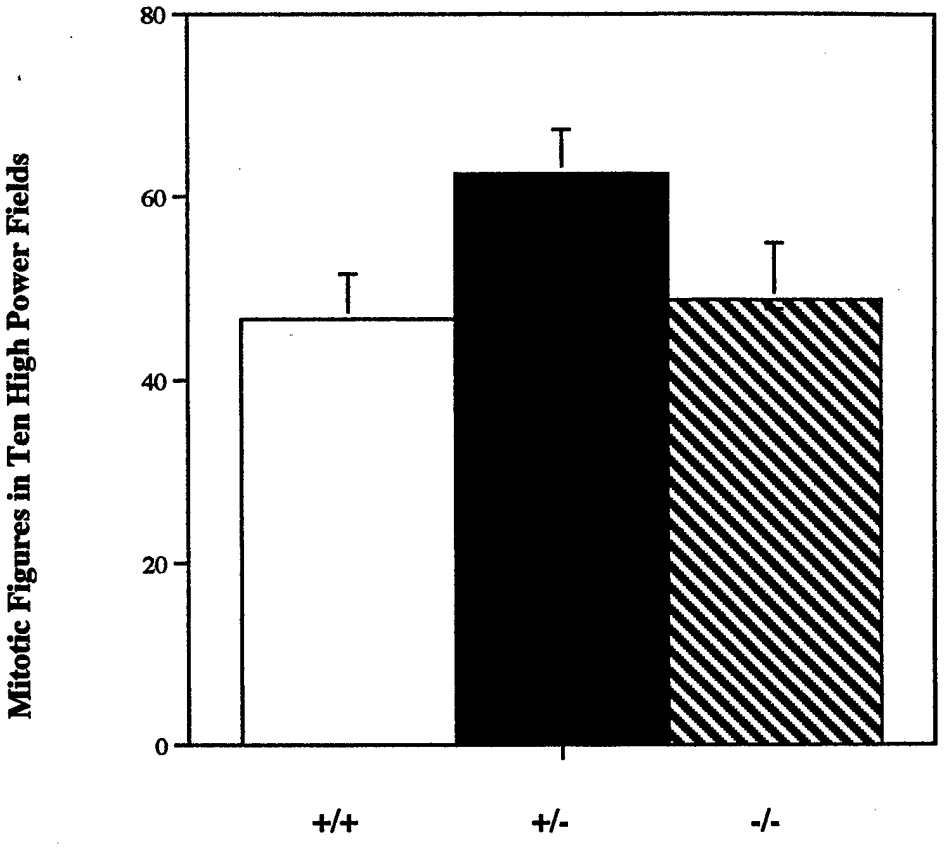


Figure 6.

Note again that the p21+/- tumors appear to have the highest mitotic rates. No differences in apoptosis rates were observed among the tumor types, indicating that the growth rate differences were driven by proliferation rather than cell death. These results were a bit surprising since it was expected that absence of p21 altogether might have an accelerated effect on tumor growth as well. However, the recent paper by LaBaer et al. (37) provides a potential explanation. High levels of p21 inhibit the kinase activity of cyclin/cdk complexes, while the absence of p21 is important in assembly of the cyclin/cdk complexes. However, low to intermediate levels of p21 appear to be sufficient to allow assembly of the cyclin/cdk complexes without being inhibitory to kinase activity of the complex. The apparent result is enhanced cell cycle progression in the presence of reduced p21 (as is observed for the p21+/- tumors).

(4) Results in Fourth Year of Grant

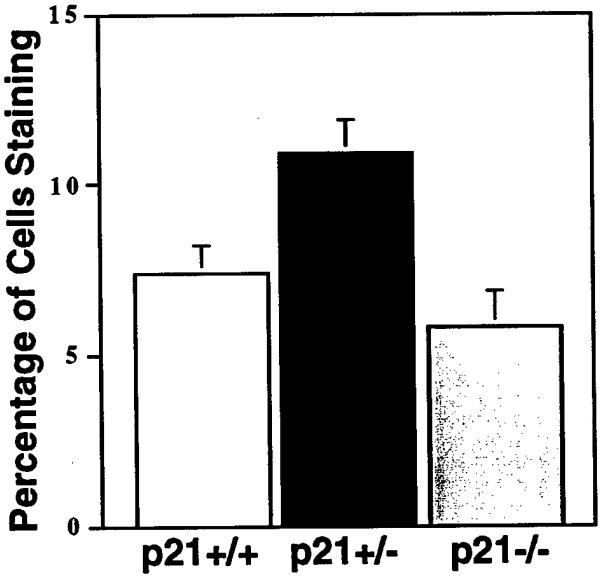
(a) Further studies of the p21-deficient *Wnt-1* transgenic tumors

In the fourth year, we continued the biological and molecular characterization of the *Wnt-1* transgenic p21-deficient tumors. The dramatically increased growth rates observed for the p21+/- tumors might be due in part to selective loss of the remaining wild type p21 allele. Such wild type allele loss occurs frequently in p53+/- tumors and results in accelerated tumor growth rates (25, 49). To assess p21 allele status in the p21+/- tumors, we performed Southern blot hybridization analysis on DNA from 17 p21+/- tumors utilizing a murine p21 probe (Figure 7). The results indicated that all p21+/- tumors retained an intact p21 allele. In fact, one tumor (arrow) exhibited loss of the mutant p21 allele, suggesting that there may be a selective advantage in retaining a single wild type p21 allele during tumorigenesis.

Figure 7. p21 allele status in *Wnt-1* transgenic p21+/- mammary tumors.

To further confirm the increased proliferation levels observed in p21+/- tumors, BrdU incorporation assays were performed. The BrdU incorporation assay assessed the percentage of tumor cells which were actively replicating their DNA over a three hour labeling period. Tumor bearing animals were injected with BrdU three hours prior to sacrifice. Following sacrifice, fixed tumor sections were prepared and subjected to standard immunohistochemical detection methods using an anti-BrdU antibody. p21+/- tumors exhibited on average 10.9% of cells labeling (n=13) while the p21+/+ and p21-/- tumors exhibited 7.4% (n=15) and 5.8% (n=9), respectively (Figure 8). Statistical analyses indicated that p21+/- tumors exhibited significantly higher levels of cells in S phase than either p21+/+ tumors (p=0.01) or p21-/- tumors (p=0.004). The results of this assay are consistent with the results of the mitotic figure counts in showing an increase in cell proliferation levels in the p21+/- tumors compared to the p21+/+ and p21-/- tumors.

Figure 8. BrdU assays on *Wnt-1* transgenic mammary tumors.



Tumor growth rates can be affected by both rates of cell proliferation and cell death. Since the absence of p21 has been shown to sensitize cells to apoptosis in a number of other model systems (54-56), it was important to determine whether the observed differences in tumor growth rates were due largely to alterations in rates of cell proliferation rather than cell death. In order to determine apoptotic levels in the p21/*Wnt-1* tumor samples they were stained immunohistochemically by TUNEL assay. As shown in Figure 9, the levels of apoptosis in the mammary tumors were very low, typically around 1% and there was no p21-dependent variation in tumor cell apoptotic levels.

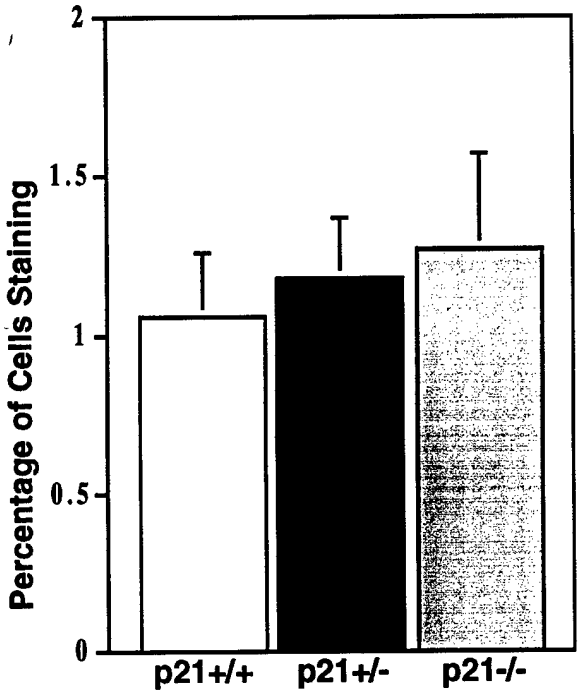
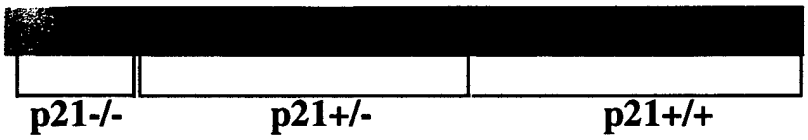


Figure 9. Levels of apoptosis in *Wnt-1* transgenic mammary tumors in the presence and absence of p21.

We hypothesized that the increased proliferation observed in the p21+/- tumors was a function of reduced levels of p21 protein associated with the G1 cyclin/cdk complexes. This reduced association might result in increased phosphorylation of Rb and an increased likelihood of entry into S phase in the p21+/- tumor cells. To test this hypothesis, we performed immunoprecipitation-Western blot assays. The p21+/+, p21+/-, and p21-/- tumor lysates were first immunoprecipitated using an anti-cyclin D1 antibody. The levels of p21 protein bound to the cyclin D1 complexes was assessed by performing Western blot analysis (with an anti-p21 antibody) of the complexes isolated by immunoprecipitation (Figure 10A). Levels of p21 present were quantitated via densitometry. Though there was variability in individual tumor samples, when the p21 levels of multiple tumors were averaged according to p21 genotype, the p21+/+ tumors exhibited approximately two-fold more p21 bound to the cyclin D1 complex than did these same complexes from p21+/- tumors (Figure10B). As expected, no p21 was observed in the p21-/- tumors. Western blot analysis of the filters using cyclin D1 antibodies revealed similar levels of cyclin D1 in all of the lanes, indicating that equal amounts of immunoprecipitate were assayed in the Western blots (data not shown). These results indicated that the p21+/- tumors did contain significantly less p21 in their cyclin D1-associated complexes than did the p21+/+ tumors.

A



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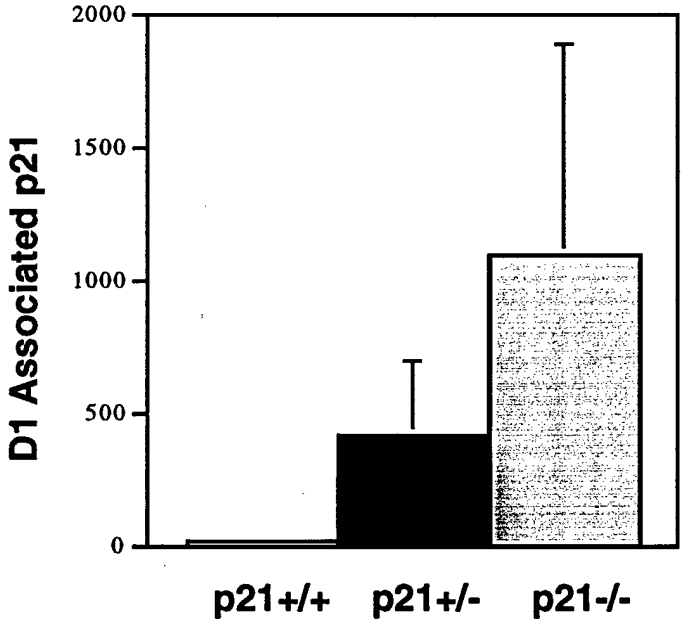

Figure 10. Cyclin D1-associated p21 in mammary tumors of different p21 genotype.

Reduced levels of p21 were associated with cyclin D1/cdk complexes in the p21+/- tumor cells compared to p21+/+ tumor cells. If so, it might be expected that the p21+/- cyclin D1/cdk complexes would exhibit higher levels of intrinsic kinase activity. To test this, we immunoprecipitated the lysates from p21+/+, p21+/-, and p21-/- tumors with an antibody to cyclin D1. The immunoprecipitated cyclin D1 complexes were then used to phosphorylate an Rb substrate as described in the Materials and Methods. As can be seen in Figure 11, complexes immunoprecipitated from p21+/- tumors exhibited on average at least two-fold higher activity than complexes prepared from either p21+/+ or p21-/- tumors. While there was considerable variability among the tumors, the mean Rb kinase activity averaged from ten tumors of each p21 genotype show statistically significant increases in the p21+/- tumors. Control reactions without Rb substrate or immunoprecipitations with antibodies to non-cyclin/cdk proteins failed to generate phosphorylated bands of the appropriate size (data not shown).

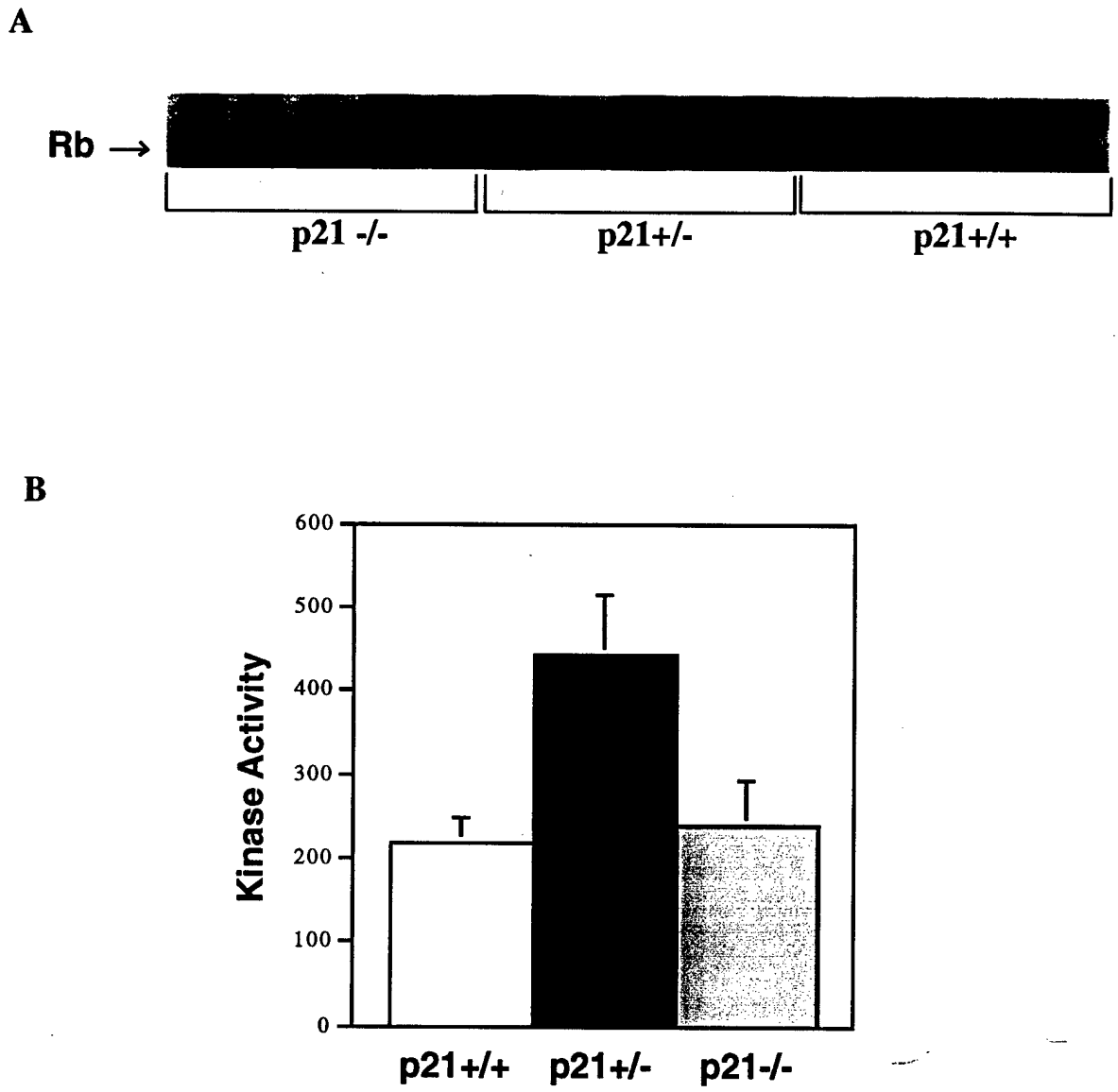

Figure 11. Cyclin D1-associated Rb kinase activity in Wnt-1 transgenic mammary tumors of different p21 genotypes.

(b) Differential gene expression in p53+/+ and p53-/- *Wnt-1* transgenic tumors

In the third year experiments described above, we initiated differential display PCR experiments to determine whether *Wnt-1* transgenic p53+/+ and p53-/- tumors had different gene expression patterns. We had found some differentially expressed fragments. In the fourth year, we began to identify and characterize these differentially expressed genes. To supplement this approach, we also made labelled cDNA probes from total mRNA populations of p53+/+ and p53-/- *Wnt-1* mammary tumors and

hybridized these to cDNA arrays obtained from Clontech. The arrays contained 588 known gene cDNAs, including a number of oncogenes, tumor suppressor genes, and other growth regulatory genes. Following probing these arrays with labelled cDNAs from several p53+/+ and p53-/- tumors, we identified several genes which were differentially expressed in a consistent manner. We confirmed the differential expression by Northern blot hybridization analysis of the candidate probe to blots containing mRNA from 8 p53+/+ and 8 p53-/- tumors (with GAPDH hybridization as a normalization standard). These combined approaches so far have revealed at least seven differentially expressed known genes in the p53+/+ and p53-/- mammary tumors. These genes were (1) cytokeratin 19, (2) kappa casein, (3) *c-kit*, (4) alpha smooth muscle actin, (5) zebrin, (6) KET, and (7) p21, which were all upregulated in the p53+/+ tumors compared to the p53-/- tumors. In addition to Northern blot analyses, where commercial antibodies are available, Western blot analyses have confirmed the p53-dependent upregulation of these genes at the protein level. A representative Northern and Western blot analysis is shown below for alpha smooth muscle actin.

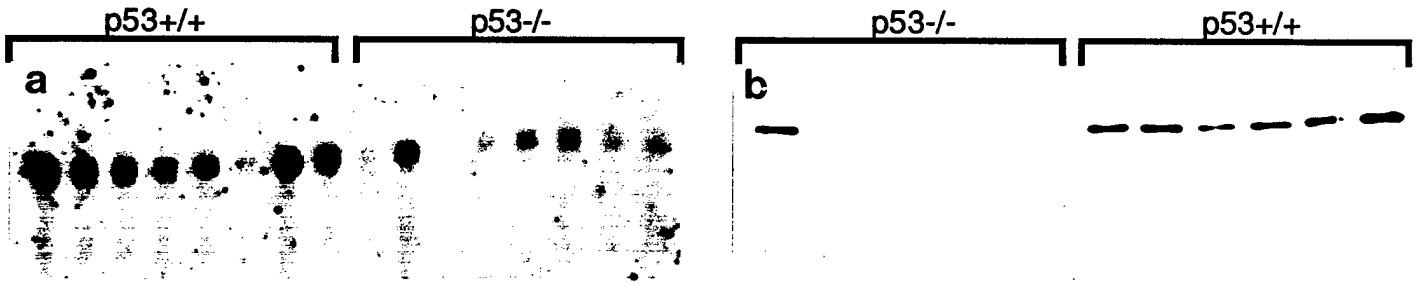

Figure 12. Alpha smooth muscle actin expression in *Wnt-1* p53+/+ and *Wnt-1* p53-/- tumors. (a) Northern blot analysis. (b) Western blot analysis with an anti-smooth muscle actin antibody.

Interestingly, many of these genes have the attributes of differentiation markers, consistent with the fact that p53+/+ mammary tumors have a much more differentiated, organized appearance compared to that of p53-/- tumors, which appear less differentiated and more anaplastic (25). Of particular interest are four of the genes, alpha smooth muscle actin, *c-kit*, KET, and p21. The alpha smooth muscle actin gene has been shown recently to be transcriptionally activated by p53 and contains a p53 response element (57). The *c-kit* gene is expressed at relatively high levels in normal human breast tissue and benign breast tumors, but downregulated or absent in malignant high grade breast tumors (58). KET is a recently identified p53-related gene which has a relatively restricted expression pattern in normal tissues (highest expression is in tongue keratinocytes) (59). Finally, increased p21 levels in the p53+/+ tumors is consistent with the lower cell proliferation levels observed in those tumors.

CONCLUSIONS

The data presented in this report argue that p53 loss or absence may contribute to tumorigenesis by a variety of different mechanisms. Tumors without p53 appear to grow and progress faster than tumors which contain p53. Surprisingly, while other mouse tumor models have demonstrated that loss of p53 is accompanied by attenuated apoptosis (16-18), we have shown in our model that apoptosis may have little or no role in tumor progression. The reasons for the apoptotic independence in our model are unclear but it may have to do with the growth factor environment of the nascent tumor cells. *In vitro* assays often show that apoptosis can only be induced in the absence of certain growth factors. The nascent *Wnt-1*-initiated tumor cell may be bathed in Wnt-1 growth factor and this may prevent any apoptotic pathways from being activated. However, this apoptotic independence makes our model particularly useful in teasing out other p53-dependent mechanisms which may influence tumor progression.

In the first two years of this grant we attempted to identify some of these alternative mechanisms. Among these are increased genomic instability in the absence of p53 and increased proliferative capacity. Interestingly, while the tumors with the most genomic instability, the p53+/- (LOH) tumors, have the highest proliferative rates, they do not arise any sooner than p53+/+ tumors. This suggests that genomic instability plays a role in tumor progression but not tumor initiation. In addition, tumor cells missing or losing p53 appear to have a higher percentage of cells in S phase, suggesting that p53 may play a direct role in decreasing tumor growth rate through its growth arrest function (through checkpoint inhibition of progression through G1 and G2 or through direct inhibition of DNA replication).

We also examined the effects of p53 loss on telomere function in the presence and absence of p53 in our mammary tumor model. Telomeres themselves appear to maintain their structure during tumor progression, though telomerase activity is greatly increased. However, this telomerase increase appears to be mostly p53-independent, arguing against a role for p53 in this particular aspect of the tumorigenesis process.

Through serendipity, we discovered that *Wnt-1* mice lacking p53 have greatly reduced amounts of heavy and light chain antibodies deposited in their tumors than *Wnt-1* mice with intact p53. The *Wnt-1* p53-/- mice also seem to be less able to mount an immune response to tumor antigens that *Wnt-1* p53+/+ mice are quite capable of recognizing. Such results argue that immune surveillance of nascent tumors in mice lacking p53 may be deficient and this may be part of the reason for the accelerated tumor incidence in *Wnt-1* p53-/- mice.

In the third year of the grant we extended our work on the *Wnt-1*/p53 model and have attempted to detect a p53-dependent effect on angiogenesis in the mammary tumors. However, we have failed to detect significant differences in the degree of vascularization of p53+ and p53- tumors, suggesting either that p53 does not affect angiogenesis in this model or that it does have a rate limiting effect, but only in the early stages of tumor initiation and formation. If so, then it would be difficult for us to discern this early effect by looking at end stage tumors.

Since the p53-dependent effects on tumor progression in our model are primarily related to cell proliferation, we decided to explore this mechanistically by investigating the role of p21 in this process. Since p21 is induced by p53 and retards cell cycle progression through binding cyclin/cdk complexes, it is a natural candidate for investigation of p53-dependent mechanisms of tumor cell proliferation. When we crossed the p21 knockout mice to the *Wnt-1* transgenic mice, we found that the *Wnt-1* transgenic females developed tumors at about the same incidence regardless of p21 status. However, only the p21+/- *Wnt-1* females exhibited fast growing tumors. This dosage effect of reduced p21 is consistent with the stoichiometric model proposed by Harlow and colleagues (37) in which high levels of p21 (e.g. in p21+/+ tumors) inhibit the kinase activity of the cyclin/cdk complex, but that low levels (e.g. in p21+/- tumors) can stimulate the complex activity. Moreover, since p21 appears actually to be an important assembly factor for some cyclin/cdk complexes, its complete absence (e.g. in p21-/- tumors) may actually be inhibitory for cell cycle progression. Corroboration for this model was provided by the demonstration that cyclin D1-associated p21 was reduced in p21+/- tumors yet these tumors had the highest levels of Rb kinase activity.

Our p21 model clearly shows that a reduction of p21 dosage can influence tumor formation and/or progression in the presence of other initiating oncogenes. Tumor growth rates are affected in a p21-dosage dependent manner. The dependence of p21 on other oncogenic events to exhibit its effects on tumor growth places it more in the category of a modifier gene than a tumor suppressor gene. Modifier genes, as exemplified by the *Mom-1* gene affecting intestinal tumor rates in *Apc*-deficient mice (60), can affect tumor formation and progression only when another tumor-initiating mutation (e.g. an *Apc* mutation) has occurred. Independent of such initiating mutations, a modifier has no apparent tumor initiating or promoting effect. Given the modification of tumor progression observed following reduction of p21 dosage, and the absence of tumors in p21-/- or p21+/- mice, we propose that p21 may be a tumor modifier gene rather than a tumor suppressor gene.

In summary, in the four year period of this grant, we have attempted to more fully answer mechanistic questions about the role of p53 loss in mammary tumor progression in our *Wnt-1* p53 model. We have shown that increased genomic instability and increased cell proliferation rates (independent of apoptosis) accompany loss of p53. Through collaborative efforts we have also examined telomere status and immune function in our model. The roles of angiogenesis and p21 in the tumorigenesis process have also been explored in the last two years. We do believe that the results achieved so far validate the *Wnt-1* transgenic p53-deficient mouse as a highly useful model for these types of studies.

Efforts to Fulfill the Statement of Work:

AIM 1: FURTHER CHARACTERIZATION OF TUMORIGENESIS IN THE p53/*Wnt-1* MICE

COMPLETION OF TUMOR BIOLOGY STUDIES OF p53/Wnt-1 MICE
1. TUMOR GROWTH RATES - Completed
2. TUMOR CELL APOPTOTIC RATES - Completed
3. IN VIVO TUMOR CELL PROLIFERATION RATES - Completed
4. IN VITRO TUMOR CELL PROLIFERATION RATES - Completed
5. INVASIVENESS AND METASTASIS ASSAYS -Completed
6. TUMOR ANGIOGENESIS ASSAYS - Completed
7. TELOMERE, TELOMERASE ASSAYS - Completed

Aim 1 Comments:

This specific aim has been completed and the results have been published (25,49,50). The invasiveness/metastasis assays and angiogenesis assays have been largely negative in showing any difference between p53+ and p53- tumors and thus these approaches have not been pursued further.

AIM 2: FURTHER BIOLOGICAL STUDIES OF CO-FACTORS WHICH INFLUENCE TUMORIGENESIS IN p53/Wnt-1 MICE

CHARACTERIZATION OF RNA EXPRESSION PATTERNS OF KNOWN GENES WHICH MAY INFLUENCE TUMORIGENESIS IN THE PRESENCE AND ABSENCE OF p53
1. p21 -completed
2. Apoptosis-associated genes - not attempted
3. Angiogenesis-associated genes - not attempted

Aim 2 comments:

So far, we have placed most of our efforts in studying the role of p21 in affecting tumor progression through the p21-*Wnt-1* crosses. Since biological assays to detect differences in apoptosis and angiogenesis between p53+/+ and p53-/- tumors failed to detect any effects, we have not placed a high priority on investigation of genes associated with these processes.

AIM 3: IDENTIFICATION OF OTHER ONCOGENE AND TUMOR SUPPRESSOR GENE LOCI ASSOCIATED WITH MAMMARY TUMORIGENESIS

DIFFERENTIAL DISPLAY TO IDENTIFY NOVEL GENES DIFFERENTIALLY EXPRESSED IN THE PRESENCE AND ABSENCE OF p53
In Progress

ISOLATION AND CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES
In Progress

cDNA ARRAY TECHNOLOGY TO IDENTIFY NOVEL GENES DIFFERENTIALLY EXPRESSED IN THE PRESENCE AND ABSENCE OF p53
In Progress

Aim 3 Comments:

We have made good progress on this specific aim, having screened 90 primer pairs in the differential display procedures and identifying 20 differentially expressed candidate fragments. We have supplemented the differential display assays with cDNA array technology. Initial screening of a number of candidate genes by Northern blot analysis has revealed at least seven differentially expressed genes.

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